THE FATE OF INDIA INK INJECTED INTRACARDIALLY INTO THE OYSTER, OSTREA VIRGINICA GMELIN

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The contributions of Metchnikoff and others to the problem of the role of the phagocyte in the "defense" reactions of invertebrates as well as vertebrates is well summarized in the book, "L'immunité dans les maladies infectieuses" (1901). His concept of the relationship between intracellular digestion in the lower forms of life and phagocytosis in higher forms of organisms still merits the attention of those interested in the evolutionary aspects of the origin of "defense mechanisms." Indeed, the role of the amebocyte in the digestive functions of the oyster has been outlined by several workers (Vonk, Yonge, Takatsuki, Nelson, Chestnut) and its role in "defense" suggested by recent findings of Nelson (personal communication) and Stauber (1945). No careful delineation of the role of the amebocytes[2] of the oyster as a "defense" cell has yet come to the author's attention. Takatsuki's paper on the nature and functions of the amebocytes of *O. edulis* is of real value but stressed the digestive aspects though several pertinent findings of his will be discussed later. On the contrary, the phagocytosis of the gymnospores of the gregarine, *Nematopsis ostrearum* Prytherch, by the amebocytes of the oyster *O. virginica* and the subsequent development of the parasites within these cells is clear evidence of their failure in this case to act as defense cells. This does not constitute proof, however, that the amebocytes are not active in defense since examples might be chosen for citation of phagocytic cells being (1) host cells for parasites (Leishmania in man or hamster, Meleney, 1925; and Sphaerita in commensal protozoa, Wenrich, 1944), (2) disseminators of stages of parasites resistant to digestion (anthrax spores in frog lymphocytes, Trapeznikoff, 1891), (3) host cells for one stage in the life cycle and not for the other stage (cryptozoic *vs.* erythrocytic stages of avian malarias, Huff and Coulston, 1944), or (4) host cells in one anatomical or physiological situation and not in another (zoochlorellae in Tridacna, Yonge, 1944). The cases cited do not constitute proof that intracellular digestion is an impossible achievement in the phagocytic cells of the organisms mentioned.

The use of injected dyes greatly aided investigators in describing the phagocytic cells and their role in local and general inflammatory processes. The papers of Buxton and Torrey (1906) are an especially interesting series of studies on the disposition and fate of metabolizable as well as non-metabolizable particulate matter injected into mammals. Cuenot's report (1914) on the phagocytic organs of molluscs lacks any mention of the oyster. Furthermore, although he used parenteral injections of particulate matter like ink, he was concerned only with their

[1] The author is deeply indebted to Dr. T. C. Nelson for his many suggestions and generous support of this work.

[2] Amebocyte, leucocyte and phagocyte are used interchangeably. The cells under discussion are found free in the blood and are both amoeboid and phagocytic.

immediate removal from the circulation, not their ultimate disposal by the mollusk. Takatsuki (1934) studied the fate of ink and carmine injected into the body of *O. edulis*. His findings, in general, are in agreement with those in the present report. Unfortunately, he fails to include in his report the details for area and amount injected, time and temperature relationships, etc.

Ranson's (1936) findings were based on the exposure of whole living oysters to a sea water medium containing neutral red, or emulsions of aniline oil and other oils or coal tar. Since the absorptive surface in these cases was apparently the whole of the exposed epithelial layer of the oyster, even though he describes a leucocytosis and mass migrations of leucocytes across epithelial layers, his observations are clearly corollary to the general object of the work begun here.

The present study was undertaken as the first step in learning the nature, extent and action of the "defense" processes in the oyster. The results reported herein constitute a clear-cut demonstration of the role of the oyster amebocyte, or phagocyte, in the disposal of large masses of particles of india ink following intracardial injection. Subsequent steps should include studies using similar injections of non-pathogenic and, if possible, pathogenic (for the oyster) organisms as well as the studies of the responses of the oyster to the local parenteral injection of a similar series of particulate matters, non-living and living.

Materials and Methods

A group of oysters was filed down with a wood rasp on the area of the left valve over the pericardium. When the shell had been worn through by the rasping, the pieces of thin shell were picked away with forceps exposing the whole pericardium. This technique was previously found to leave an undamaged oyster (Stauber, 1940) capable of living and functioning for a long time in an aquarium. The filed oysters in this series were held in the aquaria a long enough period for the mantle to lay down a thin prenacreous layer over the area exposed.

On the day of injection the new prenacreous material was picked away. Since preliminary experiments had demonstrated that the heart could not be hit through the intact pericardial wall on every occasion, the pericardium was opened to expose the heart for injection. A dropping pipette with a finely drawn out tip was used for the injections. This was partly filled with a 1 : 10 dilution of Higgins india ink in sea water and inserted into the ventricle of the oyster. Partly by the warmth of the author's fingers and partly by pressure on the bulb, amounts of diluted ink from 0.25 to 0.45 ml. were injected. The rule was to inject as much as possible under the conditions of the experiment. The injection period usually was terminated when the oyster heart became so firmly contracted that further injection of material caused escape of the ink through the puncture wound.

For the type of study reported here and because of the difficulty of estimating the weight of the oyster meat in the intact animal, no attempt was made to quantitate the mass injected on the basis of the body weight of the oyster. Since india ink precipitates in sea water, loose aggregates of various sizes were injected, some macroscopically visible. Every attempt was made to reduce these in size by shaking before injection but often reagglomeration took place in the injection pipette during the period of injection. The question of size of particle injected may be of great importance in the immediate distribution of ink observed and probably further

trials should be run using other materials for injection with more nearly uniform, smaller-sized particles for comparison (see Cuenot, 1914). Though the general picture reported here would be altered somewhat by the particle size introduced, it seems probable that the chief difference would have been an earlier, more widespread distribution of the ink.

After intracardial injection the oysters were returned to aquaria until the appropriate interval had passed (Table 1). They were then opened, the meat carefully shucked out and placed whole in fixative (Zenker-formol 5 per cent) for approximately 15 minutes, then removed to a glass plate and carefully divided into portions by cutting in the transverse plane at right angles to the long axis of the oyster. The pieces were then placed in suitable amounts of fresh fixative for 24 hours before washing. After mounting in paraffin, sections were cut at 7 μ and stained with hematoxylin and eosin.[3]

TABLE 1

Record of amounts of ink injected intracardially into the oysters and the length of interval between injection and killing time

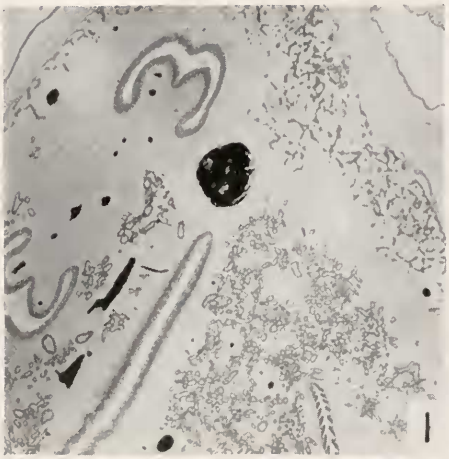
Oyster No.	Am't of india ink injected	Interval between injection and fixation
1	0.25 ml.	15 min.
2	0.3	1 hour
3	0.45	2 hours
4	0.25	4 hours
5	0.4	22 hours
6	0.25	8 days
7	0.25	17 days
8	0.25	25 days
9	0.45	33 days
10	0.25	42 days

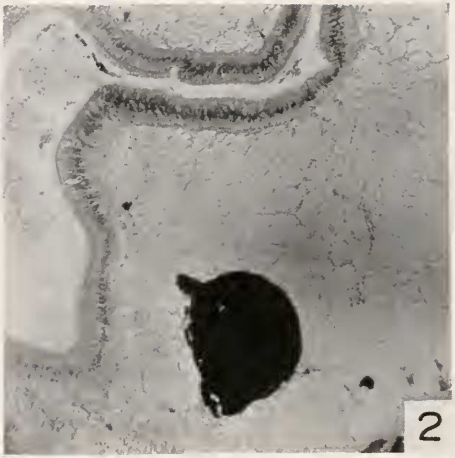
The water temperatures in the aquaria in which the injected oysters were held varied more or less directly with outside temperatures during the period. Since the period covered by the investigation was from April 8 to May 20 water temperatures in the aquaria rose irregularly from 12° to 21° C.

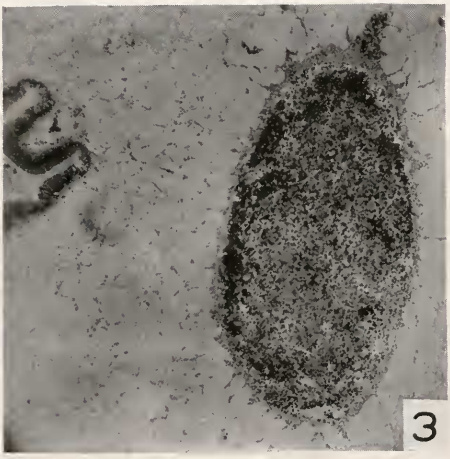
In studying the tissues the several sections of each oyster were studied microscopically, notes being made concerning the distribution of the ink both intracellularly as well as extracellularly, under the medium power as well as under the oil immersion objective. When the whole series had thus been observed key locations were critically reviewed for each oyster. The types of information tabulated may be grouped in three categories: (a) the presence or absence of ink in blood vessels or sinuses of representative areas, (b) migrations of phagocytes containing particles of ink through representative epithelia and (c) presence or absence of ink in various lumina and cavities. The following blood channels were sampled: (1) the ventral pallial artery of the mantle, (2) a sinus of the same area of the mantle, (3) the anterior aorta seen in a typical section through the visceral

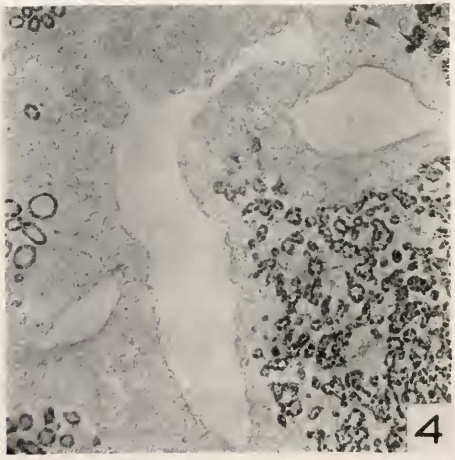
[3] The author is indebted to Miss Deborah Jackson and to Dr. James Shannon, of the Squibb Institute for Medical Research, for preparation of these tissues for microscopic examination.

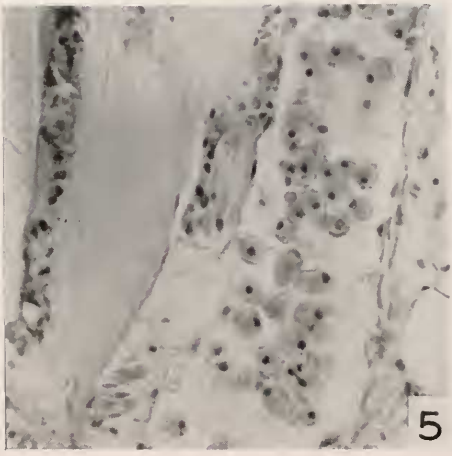
PLATE I

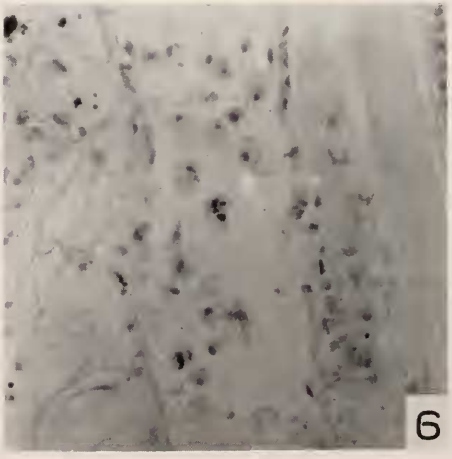












mass, (4) the largest sinus in the visceral mass cross section, (5) a small artery of the visceral mass, a subbranch of one of the hepatic arteries approximately 0.1 mm. in diameter, (6) a small sinus of the same area and magnitude for comparison, (7) an artery in the adductor muscle, (8) one of the posterior gastric arteries in the region of the so-called oral process ventral to the adductor muscle, (9) the heart, (10) the medial gill axis sinus, (11) a lateral gill axis sinus and (12) two vertical gill vessels, one leading from the medial aspect of the inner demibranch toward the medial gill axis sinus and the other from the lateral aspect of the same demibranch to the lateral gill axis sinus. In any area wherever enough phagocytes could be seen (50–100) percentage counts of those containing ink granules were recorded.

Migrations of ink-laden phagocytes through epithelial layers were sought by comparing equal numbers of representative fields from similar areas of the anatomy of the oyster. Except where noted, five consecutive fields were examined for migrating ink-laden phagocytes. The following sites were chosen as representative: (1) mantle epithelium facing the outer palp, (2) outer palp epithelium on side facing the mantle, (3) medial aspect of outer palp facing the adjacent surface of the inner palp on the same side, (4) lining of the promyal chamber near the point where it becomes contiguous with the suprabranchial chamber, (5) lining of the stomach in the area of its greatest diameter, (6) mid gut epithelium in three other locations, two areas in the visceral mass proper, and one in "oral process," (7) epithelium in the rectum dorsal to the adductor muscle, (8) a series of ten cross sections of tubules of the digestive diverticula, (9) gonaducts in region of visceral mass, (10) nephridial tubules, (11) external lining of the heart and (12) internal lining of the pericardium.

Ink, either free or in phagocytes, was sought in the lumina of five portions of the digestive tract mentioned above, in the lumina of the ten tubules of digestive diverticula examined, in the promyal chamber, in the portions of the infrabranchial

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Plate I

Figures 1–6.

Figure 1. Oyster No. 3. 2 hrs. after injection. Section through anterior end of visceral mass near the mouth. Anterior aorta and many smaller arteries are occluded with ink. Note absence of ink in large and small sinuses. × 15.

Figure 2. Oyster No. 1. 15 min. after injection. Section through tip of visceral mass ventral to the heart. Large and small arteries near the style sac are shown occluded with ink. × 50.

Figure 3. Oyster No. 6. 8 days after injection. Section through anterior end of visceral mass. Large aorta occluded by leucocytes laden with ink. × 50.

Figure 4. Oyster No. 9. 33 days after injection of ink. Section through anterior end of visceral mass dorsal to palps. Anterior aorta and a large visceral sinus shown. Neither contains any significant numbers of leucocytes. × 50.

Figure 5. Oyster No. 4. 4 hours after injection. Section through outer left lamella of outer left demibranch showing a vertical gill vessel. No ink seen in amebocytes; the single dark body in each amebocyte is the nucleus. × 625.

Figure 6. Oyster No. 6. 8 days after injection. Section through the inner lamella of the left inner demibranch near the gill axis and flanking the left suprabranchial chamber. A vertical gill vessel is shown containing ink-laden amebocytes. Similar ink-laden cells are also seen extravascularly. × 550.

chamber between palp and mantle and between the palps, in the lumina of nephridial tubules and gonaducts and in the pericardial cavity.

Results

The response of the oyster to the intracardial injection of india ink, under the conditions listed above, may be conveniently divided into four phases: arterial occlusion by the agglomerated masses of ink, phagocytosis of the ink particles, distribution of the ink by the phagocytes and elimination of the ink from the oyster. It is important to note that there was a considerable overlapping in timing of the sequence of these events.

Arterial occlusion. The immediate effect of the intravascular injection of agglomerating particles of india ink is virtual embolism of the arterial system of the oyster. The arterial system as reported by Leenhardt (1926) and Elsey (1935) is clearly outlined in this way. Even while injecting the suspension of ink the anterior and posterior aortae are seen. The arterial branches and sub branches to visceral mass (hepatics and posterior gastrics and anterior ventral), palps, mantle, adductor muscle and rectum are very noticeably blocked in the gross as well as in section with aggregations of free ink particles (Figs. 1, 2 and 9). If the lacunae, or sinuses, in areas near the arteries are examined they are usually found relatively free from blood cells and except for an occasional free granule or two, no ink is to be seen (Fig. 1). The gill axis sinuses are likewise devoid of cells and ink. Other gill vessels may show more blood cells but little or no ink (Fig. 5). The arterial emboli are first composed of ink alone (Fig. 2). From the 22nd hour to 8 days after the injection of the ink the emboli are continued by masses of phagocytes most of which have engulfed particles of ink (Fig. 3). Later, between the 8th and 17th days, with the dispersal of the ink-laden phagocytes the arteries appear as free from cells (Fig. 4) as do those of untreated oysters.

Phagocytosis. No unquestioned phagocytosis of ink was noted at the 15 min. interval (oyster No. 1) though it would easily have been obscured in the ink-filled arteries. Little or no phagocytosis was noted anywhere before the fourth hour (oyster No. 4). Occasionally, as in the gill axis vessels of oyster No. 3 at 2 hours, where numerous blood cells were seen, 1–2 per cent contained a few granules of ink. Elsewhere in the same oyster phagocytes with ink were even rarer.

Beginning with oyster No. 4 more phagocytes were seen in the arteries and more contained ink, though the distribution may be variable. For example, there were many phagocytes containing ink in arteries in the adductor muscle or visceral mass but few with ink in the ventral pallial artery.

By the end of the first day after injection (oyster No. 5) mass accumulation of phagocytes in the arteries was observed. Furthermore, almost all the ink is intracellular in those phagocytes which continue the embolic obstruction of the vessels, with most of the phagocytes containing ink granules (see Fig. 3, oyster No. 6 for a similar condition). In direct contrast to this picture, although blood cells are seen in vertical and gill axis sinuses or sinuses of viscera and mantle, the percentage of phagocytes containing ink particles ranges from 2 per cent in a lateral

gill axis and a small sinus of the visceral mass of this oyster (No. 5) to 15 per cent and 19 per cent in vertical gill sinuses.

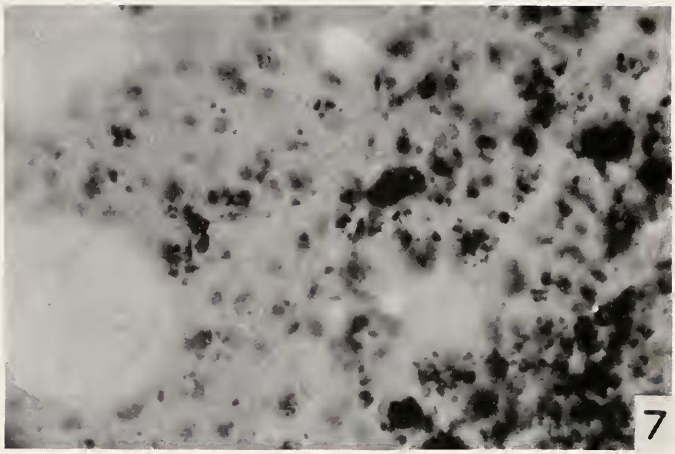
Subsequently ink is only occasionally seen free; the granules are observed almost entirely in the cytoplasm of the phagocytes. The presence of a few ink granules in blood vessels throughout the later period (oysters No. 9 and No. 10) is possibly due to its release from phagocytes upon their death or through "defecation" by active phagocytes (Chestnut, thesis), although there is no direct evidence to support this.

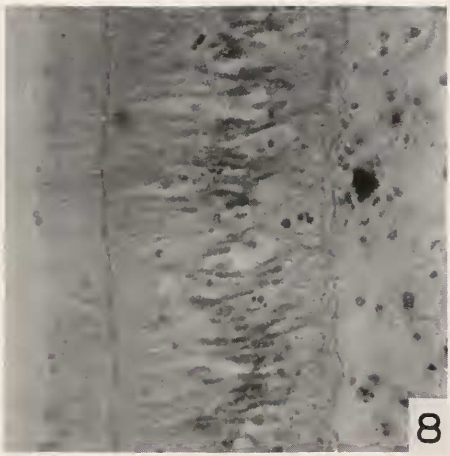
The origin of the phagocytes which engulf the large amount of ink injected is an important point to be considered. It should first be noted that even in the untreated oyster "the origin and mode of production of amebocytes are not known; no definite organ is known to produce them" (Takatsuki, 1934). It is well known and easily observed that there is a large mobile reserve of phagocytic cells. Although few of these are seen within the arteries or even in the larger blood sinuses, many are found in the smaller sinuses of gill, mantle and viscera. Probably most, however, are scattered in the smallest lacunae or intercellularly especially under the epithelium of the digestive tract, digestive diverticula and gonaducts. Their role in digestion has already been referred to and numerous migrations of phagocytic cells in the epithelia can easily be noted in oysters (Fig. 9).

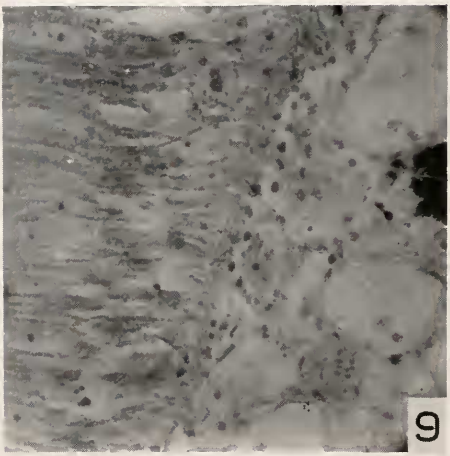
It is unlikely that mitosis of pre-existing phagocytes plays a predominate role in producing (or mobilizing) the phagocytes which dispose of the ink since, of the many phagocytes examined, only one has been observed in mitosis. This is very similar to the role of hematogenous lymphocytes and to a lesser extent monocytes as described by Maximow (1927) for the local inflammatory process in the subcutaneous tissues of the rat injected with trypan blue (Taliaferro, 1949). Since careful study of the arterial walls does not disclose mass migrations of phagocytic cells through them from the surrounding tissues their arrival at the ink masses via the hematologic route is presumed. This is understandable since, although obstruction of the artery in Figure 2 is complete, this is in an oyster *after* fixation and shrinkage. The shrinkage observed is believed not to be merely a function of the fixative used as found by Orton (1937) in his study of the effects of Bouin's fixative on isolated 0.1 gm. pieces of oyster tissues, but also of the blood loss of the cut oyster. Thus, it seems probable that blood flow did not altogether cease and that the slight trickle of fluid through these almost occluded channels made possible the appearance of the phagocytes in the ink-filled areas of the vessels. An alternative, acceptable explanation might be the migration of the phagocytes to the ink-filled arteries by their own activity. This would be possible even in a direction counter to the current (incomplete occlusion) if the current were weak,[4] and if the phagocytes moved along the blood spaces using the lining as a substrate for amoeboid activity. The usual picture of finding blood cells almost exclusively in such a position in the larger vessels may be offered as evidence, though Cuenot believed cells similarly placed on the walls of the small arteries of the "liver" of the amphineuran, *Acanthochites discrepans* to be fixed phagocytes. In the case of the oyster their relative inconstancy of number and position mark them as free phagocytes. The net result by either means would be the arrival in the blocked areas of large numbers of phagocytic cells.

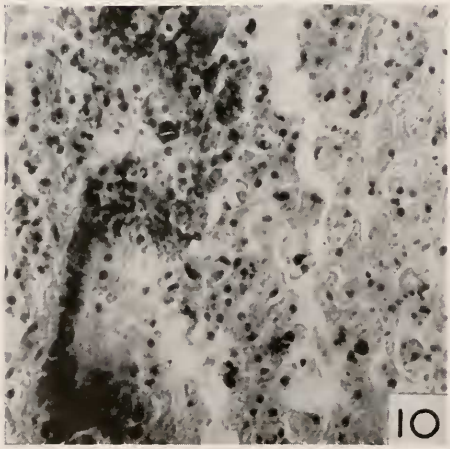
[4] The studies of T. C. Nelson (personal communication) and Hopkins (1934) show much reversal of current, much surging back and forth of blood in the vessels, especially in the mantle.

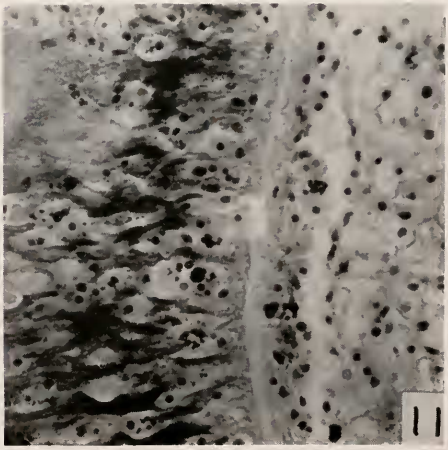
PLATE II











Takatsuki discusses the types of blood cells seen in *O. edulis* and recognizes two main types: granular leucocytes and hyaline leucocytes. No data are available on the proportionate numbers of these two types of cells in oysters or on the relative phagocytic properties of the two types of cells. Indeed, confirmation of his findings is desirable since the hyaline leucocytes are claimed to be non-nucleate and not observable in stained preparations. Since the granules in the granular leucocytes are not stained by hematoxylin-eosin the relative importance of the two cells in phagocytosis is not known, except that the only figures in Takatsuki's report which certainly identify the cell type and also show ingested particles are granular leucocytes.

Distribution. Ink-laden phagocytes become distributed throughout the oyster following engulfment in the arteries. This is accomplished in two ways: first, by the passage of the cells from small arteries to sinuses in the usual fashion, and second, by direct migration through the walls of the blocked arteries both large and small. Both processes proceed actively. For example, the large increase in the proportion of ink-laden phagocytes in the gill axis sinuses (from 2–19 per cent in oyster No. 5 to 35–50 per cent in oyster No. 6) is indicative of the first process and Figure 7 clearly shows the migration of ink-laden phagocytes through the wall of a hepatic artery in the viscera of oyster No. 6. This phenomenon was first seen in oyster No. 5 (22 hours). It was characteristic of the findings for oysters No. 6, No. 7 and No. 8 even though occlusion of the arteries was no longer to be observed in oysters No. 7 and No. 8. Some migrations were still to be observed in oyster No. 9 but the process of migration through arterial walls was virtually completed between the 33rd and 42nd days.

The net result of these processes is the widespread distribution of ink-laden phagocytes so that wherever phagocytes are found in oysters after 8 days in this series, ink-laden specimens can be observed. This is in marked contrast to the distribution of ink in a mammal, like the rat, after intracardial injection. In the mammal the ink largely remains in the organs with macrophages strategically placed to remove material from the blood (spleen, liver, bone marrow).

PLATE 11

FIGURES 7–11

FIGURE 7. Oyster No. 6. 8 days after injection. Section through wall of branch artery in visceral mass. Large numbers of ink-laden phagocytes seen in the lumen of the artery as well as many seen migrating through the wall of the artery. × 760.

FIGURE 8. Oyster No. 9. 33 days after injection. Section through wall of style sac showing migrations of ink-laden phagocytes. × 550 approx.

FIGURE 9. Oyster No. 1. 15 min. after injection. Section through style sac in approximately the same region as in Figure 8. Note the presence of a few amebocytes migrating through the epithelium of the style sac. Note also the artery occluded with ink. × 550.

FIGURE 10. Oyster No. 7. 17 days after injection. Section through palp region. To left is medial aspect of outer palp; to right is that portion of the infrabranchial chamber between the faces of inner and outer palps on the right side. Note the many ink-laden amebocytes on either side of the epithelium but especially in the infrabranchial chamber. × 600.

FIGURE 11. Oyster No. 9. 33 days after injection. Section through epithelium of intestine in the region of the oral process. Note the numerous ink-laden phagocytes migrating toward the lumen of this portion of the alimentary tract. × 550.

Takatsuki observed a similar widespread distribution of carmine-laden phagocytes in oysters injected into the body with that substance.

Elimination. The normal physiological movement of amebocytes through various epithelia is well known especially with reference to digestion (Yonge, Nelson, Takatsuki). It is not surprising then to find large scale migration of ink-laden phagocytes across epithelia into various lumina. Table II summarizes these findings. Note first that no migration of phagocytes containing ink was found until more than 22 hours had elapsed after injection of the ink. The first migrating forms with ink in them were seen in oyster No. 6 killed 8 days after injection and subsequent to the first migrations through the arteries. The epithelia of digestive

TABLE II

Migrations of ink-laden phagocytes through epithelia after intracardial injection of india ink

Oyster No.	Time after injection of ink	Number of ink-laden phagocytes observed in the epithelium†								
		Mantle, facing palp*	Palp, facing mantle*	Palp, facing palp*	Lining of promyal chamber*	Stomach*	Intestine visceral area*	Pericardial wall	Rectum*	Digestive diverticula**
1	15 min.	0	0	0	0	0	0	0	—	0
2	1 hour	0	0	0	0	0	0	0	—	0
3	2 hours	0	0	0	0	0	0	1	0	0
4	4 hours	0	0	0	0	0	0	0	0	0
5	22 hours	0	0	0	0	0	0	—	0	0
6	8 days	2	0	6	1	0	4	15	—	0
7	17 days	1	1	5	0	8	6	41	0	14
8	25 days	1	0	1	2	0	0	—	1	1
9	33 days	0	1	7	1	36	33	1	15	10
10	42 days	0	0	2	0***	20	8	6	2	9

— Area not observed.
0 No migrations observed.
* 5 oil immersion fields studied.
** 10 cross sections of tubules of digestive diverticula examined.
*** Many ink-laden phagocytes seen in the promyal chamber.
† The numbers are relative and of chief significance when compared with others in the same vertical column.

organs (stomach, intestine, rectum, digestive diverticula) were the chief sites of the migrations (Figs. 8 and 11) though the presence of many ink-laden phagocytes between the palps on one side of oyster No. 7 (Fig. 10) or in the promyal chamber of oyster No. 10 indicates that the process occurred in a wide variety of epithelial sites. Migrating phagocytes were observed in the lining of both the pericardial wall and the outer aspect of the heart as well as in the pericardial space between these sites.

There were three sites in this series which, almost without exception, showed no migrating ink-laden phagocytes. These were the epithelia of the external (shell-secreting) face of the mantle, the gonaducts and the excretory tubules. Migrations through the epithelium of the gills were relatively rare though it is possible that this is a reflection of the initial distribution of the ink. While the primary occlusion

of the arteries in the visceral mass, and the later migrations of ink-laden cells through the arterial walls of the visceral arteries, would account in part for the sites of migration observed, the failure to observe such migrations through gonaducts, excretory tubules and the external face of the mantle must be explained in some other fashion. Similarly, the presence of 35–50 per cent ink-laden phagocytes in the gill sinuses of oyster No. 6 is contrasted to the rare migrations of these cells across the gill epithelium. Takatsuki reported similar migrations of india ink or carmine-laden phagocytes across epithelia in various sites after injection of carmine into the body of the oyster, but his findings included migrations into excretory tubules and gonaducts. The discrepancy is not easily explained with the data available. Even his possible faulty interpretation through the use of the term "excretion" to include the voiding of undigested material from the body cannot explain the migrating leucocytes he observed. Nowhere in his report does he record the site of the injection of the carmine or ink. If the site had been into or in the vicinity of the excretory organ, a plausible explanation would be available; although, in this series, microscopic fields have been observed showing many ink-laden phagocytic cells just beneath the basement membrane of the epithelium of the nephridial organ without a single phagocyte in migration through the epithelium. Perhaps the frequency of migrations unrecorded by him through these sites is a partial explanation. In the series reported here, only a single ink-laden cell was observed traversing the epithelium of an excretory tubule. The absence of phagocytes from the lumen of the excretory tubules might be explained by the open pericardium since the wall of the auricle is claimed to have excretory functions (Ranson, 1936). This is especially true since we did observe ink-laden phagocytes in the pericardial cavity. In the case of the gonaduct, Takatsuki himself was at loss to explain such migrations. It is possible that his study was made in the autumn when the oyster amebocytes play an important physiological role in the involution of the gonad. Our study covered a period in the spring when the gonad was showing renewed activity. No migrations into gonaducts were observed.

The net result of these migrations was the voiding of ink into the lumina of organs leading to the outside. In fact, once the ink particles had passed through these epithelia they may be considered essentially as being "outside" of the body of the host. It is possible, of course, that some of these phagocytes (especially in the intestine and digestive diverticula and even on palp and gill surface) may migrate back into the oyster again if the ink present in their cytoplasms does not interfere with the performance of other functions. A net loss of ink would be expected, however, which would eventually lead to complete elimination.

Further evidence of this elimination was seen in the appearance in the aquaria of dejecta and rejecta of much darker appearance than that of uninjected oysters under similar conditions. Since no microscopic observations were made on these voided masses it cannot be stated whether the granules of ink were free or still in phagocytic cells when voided from the oyster. Chestnut (personal communication), after local parenteral injections of small amounts of india ink, states definitely that the ink in the feces was in phagocytes.

It will be noted from Table II that ink was still being eliminated from the oyster when the experiment was terminated, though the peak of elimination occurred on or about the 33rd day after the injection of ink. Oyster No. 10 was noted as having

less ink present than any other oyster in the series. Although there are no supporting data available it is believed that the process would continue for a relatively long period of time with the curve of elimination gradually flattening out and with some ink still present, possibly for a year or more.

Discussion

The process of elimination of india ink after intracardial injection is not confined to the oyster. Although no references to exactly similar work in mollusca have been found, vestiges of a similar mechanism for the removal of foreign bodies are described for mammals. Two related statements from Maximow and Bloom, "Textbook of Histology" (1939), are pertinent. Discussing free macrophages of the blood the authors state, "Many investigators have described macrophages in the blood. In animals injected intravenously with vital dyes or corpuscular matter,

Table III

Time sequence of events leading to disposal of india ink after intracardial injection into the oyster

Oyster number	Time after injection	Arterial occlusion	Presence of free india ink in blood vessels	Migrations of ink-laden phagocytes through arterial walls	Migrations of ink-laden phagocytes through epithelia
1	15 min.	+	++++	−	−
2	1 hour	+	++++	−	−
3	2 hours	+	++++	−	−
4	4 hours	+	+++	−	−
5	22 hours	+	+	+	−
6	8 days	+	±	++	+
7	17 days	−	±	++	++
8	25 days	−	±	++	++
9	33 days	−	±	+	++++
10	42 days	−	±	−	++

The numbers of pluses are approximate and relative and are significant only when compared with others in the same vertical column.

such as blood cells, large quantities of these substances accumulate in the free macrophages. They originate in the spleen, liver and bone marrow from fixed macrophages through contraction and isolation. They are found especially in the blood of the veins and of the right heart and the major part of them is filtered off in the capillaries of the lungs, but some may occasionally enter the general circulation" (p. 43). "If the macrophages have taken up indigestible particulate matter, as colloidal silver or india ink, they often degenerate; then the foreign particles are set free and may be taken up again by other macrophages. In the intestines and in the lungs the foreign particles may be eliminated from the body with the cells containing them" (p. 91). A local response of similar nature is described for the lungs. In this case, part of the carbon inhaled in city air is phagocytized and expelled in the so-called "dust cell," a macrophage of the lung. In the oyster, however, the process is more closely related to the intracellular phases of the digestive process.

In a poikilothermic animal, like the oyster, the rates of various metabolic processes are greatly influenced by temperature. That the degree of activity of the phagocytes would be similarly affected by temperature is a logical conclusion. Therefore, the times reported here for phagocytosis, distribution and elimination of india ink (see Table III) must be considered in terms of the temperatures prevailing during the experiment. If the experiment had been conducted at 25° C. the events noted most probably would have occurred much sooner than recorded above.

A half century ago the responses of many organisms to the injection of foreign bodies, living and dead, were studied but the oyster was not included. It is strange that the oyster, an organism of such great value as a food in many parts of the world, especially in this country, and claimed to be scientifically the best known marine animal, has not been fully studied in this respect. Only a fraction of the oyster eggs fertilized each year grow to adult oysters. Many causes of mortality are known, predators like the starfish and oyster drill being especially important. There are always found, however, losses of unknown causation (Orton, 1924; and Loosanoff and Engle, 1941). Except for Tennent's work on *Bucephalus*, Prytherch's work on *Nematopsis* and Korringa's recent report on a fungal shell disease, few diseases peculiar to the oyster are known where the parasitic organism (not predator) produces direct effects on the living substance of the oyster (Dollfus, 1921; Orton, 1937). This is not meant to imply that *Bucephalus* or *Nematopsis* is necessarily a primary cause of mortality of the oyster. It is very unlikely that these are the only ones that do exist. The beginning study reported here is believed fundamental to a full knowledge of host responses upon which control measures might be based.

A similar series of oysters should be studied using foreign body particles of much smaller size. While arterial occlusion would probably be lacking and initial distribution of the ink less restricted, the phenomena of phagocytosis, migration and elimination would most likely be similar.

In the life cycle of *Nematopsis ostrearum*, a gregarine parasite the sporozoite of which normally develops in the phagocytes of the oyster, Prytherch notes that "though some of the sporozoites are evidently destroyed by the phagocytes a small number generally survive and grow rapidly at their expense." The mortality of sporozoites in a light natural infection was estimated at better than 50 per cent. Development of the sporozoite and spore formation in the oyster require about two weeks in warm weather, according to Prytherch. It is entirely possible that, in addition to the mortality mentioned above, there is an important sporozoite loss due, not to intracellular digestion by the phagocytes, but to migration and elimination of the sporozoite-laden phagocytes as in the case of the india ink granules described above. Indeed, unless the hypertrophy of the sporozoite-infected phagocyte restricts amoeboid activity, it is possible that the elimination of developed spores in this fashion may constitute the more normal route of infection for the crab host.

Summary

The responses of the oyster to an intracardial injection of a sea water suspension of india ink were followed grossly and microscopically. The ink suspensions agglomerated readily and produced emboli which virtually occluded the arterial vessels of viscera, mantle and adductor muscle. Subsequent events, with considerable over-

lapping, were in sequence: (a) phagocytosis of the injected ink particles by mobile phagocytes, (b) distribution of the ink in the phagocytic amebocytes to all parts of the organism with concomitant resolution of the emboli and (c) eventual elimination of the ink from the organism by the migration of ink-laden phagocytes through the epithelial layers of the alimentary tract, digestive diverticula, palps, mantle, heart and pericardium into lumina from which they were voided. The epithelia of gonaducts, nephridia and shell-forming mantle were not routes of migration. A close relationship is noted between the role of the phagocytes in the normal digestive process and in the "defense" reaction to such a foreign body as india ink. The possible significance of the responses noted with respect to unexplained mortalities of the oyster is considered.

The author is deeply indebted to Dr. T. C. Nelson for taking Figures 2–4 and 7–11 inclusive.

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